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N-recognin/Ubc2 Interactions in the N-end Rule Pathway*

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The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue. In the yeast *Saccharomyces cerevisiae*, substrates of the N-end rule pathway are targeted for degradation by a complex that includes the 225-kDa N-recognin, encoded by *UBR1*, and the 20-kDa ubiquitin-conjugating enzyme encoded by *UBC2*. We report that both physical stability and functional activity of the N-recognin • Ubc2 complex require the presence of a highly acidic 23-residue region at the C terminus of Ubc2. Ubc2-C88A, an inactive variant of Ubc2 in which the active-site Cys-88 has been replaced by Ala, is shown to retain the affinity for N-recognin. Expression of Ubc2-C88A inhibits the N-end rule pathway, apparently as a result of competition between Ubc2 and Ubc2-C88A for binding to N-recognin. The two-hybrid (interaction cloning) technique was used to identify a ~170-residue C-terminal fragment of the 1,950-residue N-recognin as a Ubc2-interacting domain. We also show that the level of *UBR1* mRNA decreases upon overexpression of *UBC2*. This effect of *UBC2* is observed with cells whose *UBR1* is expressed from an unrelated promoter but is not observed if *UBR1* contains a frameshift mutation, or if the Ubc2 protein lacks its C-terminal acidic region. The N-recognin • Ubc2 complex appears to regulate the expression of N-recognin through changes in the metabolic stability of its mRNA.

Among the functions of intracellular proteolysis are the elimination of abnormal proteins and the temporal control of many cellular processes that involve short-lived regulators. Amino acid sequences, conformational determinants, or chemically modified protein structures that confer metabolic instability are called degradation signals, or degrons (Varshavsky, 1991). An essential component of one degradation signal is the protein's N-terminal residue (Bachmair *et al.*, 1986). The presence of this signal, the N-degron, is manifested as the N-end rule, which relates the *in vivo* half-life of a protein to the identity of its N-terminal residue (reviewed by Varshavsky, 1992). Distinct versions of the N-end rule operate in all organisms examined, from mammals to yeast and bacteria (Bachmair *et al.*, 1986; Gonda *et al.*, 1989; Tobias *et al.*, 1991).

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The eukaryotic N-degron comprises at least two determinants: a destabilizing N-terminal residue and an internal Lys residue (or residues) (Bachmair and Varshavsky, 1989; Johnson *et al.*, 1990; Dunten *et al.*, 1991). The latter is the site of attachment of a multiubiquitin (multi-Ub) chain whose formation is required for the degradation of at least some N-end rule substrates. In a multi-Ub chain, several ubiquitin (Ub)[1] moieties are attached sequentially to an acceptor protein, forming a chain of Ub-Ub conjugates in which the C-terminal Gly-76 of one Ub is joined to Lys-48 of the adjacent Ub (Chau *et al.*, 1989; Johnson *et al.*, 1992). A substrate that bears a multi-Ub chain is degraded by the 26 S proteasome, a ~1,500 kDa, ATP-dependent, multicatalytic protease that contains more than 20 distinct subunits. Different Ub-dependent pathways are mediated by distinct targeting complexes while apparently sharing at least a protease component (the 20 S or "core" proteasome) (reviewed by Finley and Chau, 1991; Rechsteiner, 1991; Hershko and Ciechanover, 1992; Goldberg, 1992; Hochstrasser, 1992; Varshavsky, 1992).

In the yeast *Saccharomyces cerevisiae*, the recognition component of the N-end rule pathway, called N-recognin (it is also known as E3α (Hershko and Ciechanover, 1992)), is encoded by the *UBR1* gene (Bartel *et al.*, 1990). The 225-kDa N-recognin (Ubr1) selects potential N-end rule substrates by binding to their primary destabilizing N-terminal residues Phe, Leu, Trp, Tyr, Ile, Arg, Lys, and His. N-recognin has at least two substrate-binding sites. The type 1 site is specific for the basic N-terminal residues Arg, Lys, and His. The type 2 site is specific for the bulky hydrophobic N-terminal residues Phe, Leu, Trp, Tyr, and Ile (Reiss *et al.*, 1988; Gonda *et al.*, 1989; Baker and Varshavsky, 1991). These N-terminal residues are bound directly by N-recognin, whereas secondary destabilizing N-terminal residues (Asp and Glu in yeast) function through their conjugation, by Arg-tRNA-protein transferase (R-transferase), to Arg, one of the primary destabilizing residues. Tertiary destabilizing N-terminal residues, Asn and Gln, function through their conversion, by a specific amidase, into the secondary destabilizing residues Asp and Glu (Gonda *et al.*, 1989; Balzi *et al.*, 1990; reviewed by Varshavsky, 1992).

In *S. cerevisiae*, the ubiquitination of N-end rule substrates has been found to require the Ub-conjugating enzyme encoded by the *UBC2* gene (its old name is *RAD6*), and Ubc2 has been shown to be physically associated with N-recognin (Dohmen *et al.*, 1991a). The yeast Ubc2 can also function as an N-end rule-mediating Ub-conjugating enzyme in a heterologous cell-free system such as an extract from rabbit reticulocytes (Sung *et al.*, 1991). Ubc2 is one of at least 10 distinct Ub-conjugating

[1] The abbreviations used are: Ub, ubiquitin; Nt-amidase, N-terminal amidase; kb, kilobases or kilobase pairs; βgal, *E. coli* β-galactosidase; Ub-*X*-βgal, ubiquitin-*X*-βgal, where *X* is an amino acid residue at the Ub-βgal junction; PAGE, polyacrylamide gel electrophoresis; XGal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ha, hemagglutinin.

enzymes (also called *E2* enzymes) in *S. cerevisiae* (Jentsch *et al.*, 1987; reviewed by Jentsch, 1992). The *E2* enzymes use activated Ub, produced by the Ub-activating (*E1*) enzyme, to catalyze the formation of isopeptide bonds between the C-terminal Gly-76 of Ub and ε-amino groups of lysines in acceptor proteins (reviewed by Pickart, 1988; Finley and Chau, 1991; Jentsch, 1992).

Processes that are known to be perturbed by mutations in *UBC2* include DNA repair, induced mutagenesis, cell cycle control, sporulation, regulation of retrotransposon transposition, and the N-end rule pathway of protein degradation (Reynolds *et al.*, 1985; Borts *et al.*, 1986; Friedberg, 1888; Picologlou *et al.*, 1990; Madura *et al.*, 1990; Ellison *et al.*, 1991; Kang *et al.*, 1991; Dohmen *et al.*, 1991a, and references therein). The N-end rule pathway is inactive in either a *ubc2* or a *ubr1* null mutant; however, the overall *ubc2* phenotype is more severe than that of *ubr1*, indicating that the functions of the Ubc2 enzyme are not confined to the N-end rule pathway (Dohmen *et al.*, 1991a).

While it is likely that most of the *E2* enzymes in yeast and other eukaryotes are guided to their *in vivo* substrates by *E2*-associated "recognition" subunits (recognins) (Varshavsky, 1992), the yeast N-recognin · Ubc2 complex remains the only example of such an assembly that has been defined genetically. A further biochemical and genetic dissection of this complex is described below.

EXPERIMENTAL PROCEDURES

Strains, Media, Genetic Techniques, and β-Galactosidase Assay—The *S. cerevisiae* strains used in this work are listed in Table I. *S. cerevisiae* were grown at 30 °C in rich (YPD) or synthetic media (Sherman *et al.*, 1986), with the latter containing either 2% dextrose (SD medium), 3% galactose (SG medium), or 3% raffinose (SR medium). Transformation of *S. cerevisiae* was carried out by the methods of Dohmen *et al.* (1991b) or Schiestl and Gietz (1989). Enzymatic activity of βgal in extracts from yeast cultures in exponential growth ($A_{600} < 1$) was measured using the *o*-nitrophenyl-β-D-galactoside assay as described by Baker and Varshavsky (1991).

Plasmid Construction—Standard methods were used (Ausubel *et al.*, 1989). DNA fragments were isolated from agarose gels using GeneClean (Bio101, La Jolla, CA). The high copy (2 μ-based) plasmid pADHUBC2 which expressed Ubc2 from the strong P_{ADH1} promoter was constructed using the vector YEplac195 as described in Dohmen *et al.* (1991a). The analogous plasmids pJD647, pJD617, pJD616, and pJD615 that expressed, respectively, Ubc2-C88A, Ubc2-164, Ubc2-153, and Ubc2-149 from the P_{ADH1} promoter were constructed by inserting these mutant alleles of *UBC2*, contained in *Eco*RI fragments from plasmids pR647 (Sung *et al.*, 1990), pR617, pR616, and pR615 (Morrison *et al.*, 1988) (gifts from L. Prakash, University of Rochester, NY), into *Eco*RI-cut YEplac195ADH (Dohmen *et al.*, 1991a).

The plasmid pKM1167 that expressed *UBC2* from the galactose-inducible P_{GAL1} promoter was constructed by first isolating a ~0.6-kb *Bam*HI-*Eco*RI fragment of pG12 (Nicolet and Friedberg, 1987) that contained the $P_{GAL1/GAL10}$ promoter region, blunting the fragment's ends with Klenow PolI, and ligating the fragment into the *Sma*I-cut, low copy (*CEN*-based) plasmid YCplac33 (Gietz and Sugino, 1988), yielding pKM1035. A ~0.6-kb fragment of pDG309-containing *UBC2* (a gift from R. D. Gietz, University of Manitoba, Winnipeg, Canada) was then ligated into *Eco*RI-cut pKM1035, yielding pKM1167. The *ubc2-148* allele of *UBC2*, used in *UBC2-UBR1* coexpression experiments (Fig. 4), was constructed from *UBC2* using polymerase chain reaction and synthetic oligodeoxynucleotides (Ausubel *et al.*, 1989). The polymerase chain reaction-produced fragment containing *ubc2-148* was ligated into *Eco*RI-cut pKM1035, yielding pKM1166.

To express *UBR1* from the copper-inducible P_{CUP1} promoter, we modified the high copy plasmid pJD1003, a derivative of pSOB35 that encoded Ubr1 and contained a unique *Sal*I site (produced by site-directed mutagenesis) 15 base pairs upstream of the start (ATG) codon of *UBR1*.[2] This plasmid was digested with *Pst*I and *Esp*I, treated with T4 DNA polymerase, and self-ligated, yielding pKM1307, which lacks a 5′-proximal portion of the *QCR9* gene that was present in our earlier *UBR1*-expressing plasmids. *QCR9*, located next to *UBR1* on Chromosome VII, encodes subunit 9 of the mitochondrial ubiquinol-cytochrome *c* oxidoreductase complex (Trumpower, 1990; Phillips and Trumpower, 1993). Cells carrying *UBR1*-expressing, high copy plasmids that also expressed a truncated *QCR9* had a mild petite-like phenotype (slow growth on inefficiently fermentable carbon sources such as raffinose or galactose, data not shown). The ~0.35-kb, P_{CUP1} promoter-containing region of the plasmid pJDC22-2 (see below) was excised by *Sal*I and *Sac*I, and ligated into *Sal*I/*Sac*I-cut pKM1307, yielding pKM1315, which expressed *UBR1* from the P_{CUP1} promoter. (pJDC22-2 was constructed by a multistep protocol (details available upon request) that included the insertion of a modified, P_{CUP1} promoter-containing fragment from the plasmid YEp46 (Ecker *et al.*, 1987) (a gift from D. Finley, Harvard Medical School, Boston, MA) into the *Bam*HI-cut polylinker of YCplac22 (Gietz and Sugino, 1988).) The plasmid pKM1315 was treated with *Spe*I and Klenow PolI, and self-ligated, producing a 4-base insertion/frameshift mutation at the nucleotide position 510 in the *UBR1* open reading frame (Bartel *et al.*, 1990). The resulting plasmid, pKM1320, expressed a 170-residue N-terminal fragment of the 1,950-residue Ubr1 protein (the mutation produced a stop codon immediately after the nucleotide position 510). Neither pKM1315 nor pKM1320 inhibited cell growth under conditions that induced their *UBR1*-linked P_{CUP1} promoter (0.1 mM $CuSO_4$, see Fig. 4).

Isolation of RNA and Northern Hybridization—A slight modification of the procedures described by Baker *et al.* (1992) was used. *S. cerevisiae* strains KMY633, 634 and 635 (Table I) were grown to A_{600} of ~1 in SR medium containing 0.1 mM $CuSO_4$ and lacking uracil and tryptophan. A 15-ml sample of each culture was centrifuged to pellet the cells, which were then washed with 1 ml of water. Galactose was added to the remainder of the cultures to a final concentration of 3%. Samples were withdrawn from the cultures 1, 2, and 3 h after the addition of galactose. Washed cell pellets were resuspended in 0.25 ml of ice-cold TEN buffer (0.5 M NaCl, 10 mM Na-EDTA, 0.2 M Tris-HCl, pH 7.5). An equal volume of glass beads and 0.25 ml of phenol-chloroform (1:1, equilibrated at pH 8) were added, followed by vortexing at maximum speed for 2 min, with intermittent cooling on ice (Silverman, 1987). The samples were centrifuged at 12,000 × *g* for 1 min, and the aqueous phase was reextracted twice with phenol-chloroform, followed by the addition of 2 volumes of 95% ethanol and incubation at −20 °C to precipitate nucleic acids, which were recovered by centrifugation at 12,000 × *g* for 5 min. The nucleic acid pellets were dissolved in 2 mM dithiothreitol containing 500 units/ml of RNAsin (Promega) The concentration of RNA was determined by measuring A_{260} (these preparations contained negligible amounts of DNA, data not shown).

For Northern hybridization, a slightly modified procedure of McMaster and Carmichael (1977) was used. 25 μg of total RNA was treated with 1.5 M deionized glyoxal, 50% dimethyl sulfoxide, 10 mM sodium phosphate, pH 6.5, at 50 °C for 1 h, cooled, and electrophoresed at 4 V/cm, with recirculation of electrophoretic buffer, in an 0.8% agarose gel containing 10 mM sodium phosphate, pH 6.5. The gel was incubated in 25 mM sodium phosphate, pH 6.5, for 15 min, and the RNA was transferred by blotting in the same buffer to a GeneScreen membrane (Du Pont). The gel was stained with ethidium bromide and photographed at 300 nm before and after the transfer to verify equimolarity of RNA sample loads and the uniformity of transfer. The membrane was heated at 85 °C for 3 h, placed in 7% SDS, 1% bovine serum albumin, 0.5 M sodium phosphate, pH 7.0, sealed in a plastic bag, and prehybridized for 30 min at 65 °C. ^{32}P-Labeled DNA probes were prepared by the method of Feinberg and Vogelstein (1984), using 0.1 μg of gel-purified DNA fragments. The heat-denatured DNA probes were added to the plastic bag and incubated with the membrane-immobilized RNA for ~15 h at 65 °C. The membrane was then washed twice for 30 min at 65 °C in 0.2% SDS, 0.3 M NaCl, 30 mM sodium citrate, pH 7.0.

The *UBR1*-specific probe was a ~5.3-kb *Spe*I-*Kpn*I fragment of pSOB44 (Bartel *et al.*, 1990) that is contained within the *UBR1* open reading frame. The *UBC2*-specific probe was a ~0.6-kb *Eco*RI fragment of pDG309 (see above) that included the entire 516-base pair open reading frame of *UBC2*. The probes were labeled with ^{32}P and mixed together before hybridization. After completion of the Northern analysis, a control hybridization was carried out with the same membrane (without removing the hybridized *UBR1* and *UBC2* probes), using a ^{32}P-labeled *ACT1* (yeast actin gene) probe (a ~2-kb *Bam*HI-*Pst*I fragment of pTB392; a gift of J. Jones, McArdle Laboratory, University of Wisconsin, Madison, WI). Hybridization patterns were detected either by standard autoradiography with x-ray

[2] R. J. Dohmen, B. Bartel, and A. Varshavsky, unpublished data.

films or using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) (Johnston *et al.*, 1990; Tobias *et al.*, 1991) and quantified as described in the legend to Fig. 4*D*.

Coimmunoprecipitation Assay—Cells from 20-ml exponential yeast cultures (A_{600} < 1) of the strain BBY68 (*ubc2Δ ubr1Δ*, Table I) transformed with various plasmids (see "Results") were collected by centrifugation at 2,500 × *g* for 5 min, resuspended in 0.5 ml of SD medium lacking methionine, and labeled with 0.2 mCi of [^{35}S]Translabel (ICN) for 30 min at 30 °C. The labeled cells were pelleted by centrifugation, resuspended in 0.5 ml of DB buffer (50 mM NaCl, 1 mM Na-EDTA, 50 mM Na-HEPES, pH 7.5) containing protease inhibitors leupeptin, pepstatin A, antipain, chymostatin, and aprotinin (each at 20 μg/ml, Sigma), and lysed by vortexing with glass beads (Bartel *et al.*, 1990). The extracts were centrifuged at 12,000 × *g* for 10 min. Immunoprecipitation assays were carried out with the supernatants by adding ascitic fluid (1 μl/0.3 ml of a supernatant) containing the anti-ha monoclonal antibody 12CA5 (Field *et al.*, 1988) (Babco Inc., Richmond, CA) to the single or mixed extracts described under "Results" and in the legend to Fig. 2. All samples also contained a ~10-fold excess of an identically prepared yeast extract from untransformed, unlabeled BBY68 (*ubc2Δ ubr1Δ*) cells. The samples were incubated on ice for 2 h; 20 μl of Protein A-Sepharose (Repligen, Cambridge, MA) was then added, and the suspensions were incubated, with end-over-end rotation, for 1 h at 4 °C, followed by a 5-s centrifugation in a microcentrifuge. Pellets were washed four times with 0.8 ml of DB buffer containing 5% glycerol, resuspended in electrophoretic sample buffer, heated at 100 °C for 3 min, and subjected to SDS-PAGE (13%), followed by fluorography. ^{14}C-Labeled SDS-PAGE protein standards were from Amersham Corp.

Interaction Cloning of Ubc2-binding Protein Domains—The *S. cerevisiae UBC2* gene containing an *Eco*RI site immediately 5′ to its start (ATG) codon (a gift from R. D. Gietz) was isolated as a 525-base pair *Eco*RI-*Sal*I fragment and ligated into *Eco*RI/*Sal*I-cut pMA424 (Ma and Ptashne, 1987), yielding the plasmid pKM1210–1, which expressed a fusion containing the DNA-binding domain of Gal4 upstream of the complete sequence of Ubc2. The *S. cerevisiae* strain GGY1::171 (Gill and Ptashne, 1987) was transformed (Schiestl and Gietz, 1989) with pKM1210–1. His$^+$ transformants were selected on DOBA-his plates (BIO101), yielding KMY528 (Table I). This strain was transformed with DNA pools from the three libraries pGAD1, 2, and 3, in which yeast genomic DNA fragments (produced by a partial *Sau*3A digestion) had been fused, in three different frames, to an upstream fragment that encoded the activation domain of Gal4 (Chien *et al.*, 1991; a gift from P. Bartel, SUNY, Stony Brook, NY). Transformants were selected on DOBA-his-leu plates (BIO101), and ~1.5 × 10^6 colonies were replica-plated onto the same medium containing the chromogenic β-galactosidase substrate XGal (Chien *et al.*, 1991).

This screen yielded 14 blue transformants that remained blue upon restreaking. The *HIS3*-based pKM1210–1 was evicted from these transformants by growing them on histidine-containing medium. The library-derived plasmids were isolated from the resulting His$^-$ transformants as described by Hoffman and Winston (1987) and transformed into the *Escherichia coli* strain DH5α. The amplified plasmids were purified from *E. coli* and transformed into KMY528 (Table I), confirming the results of the initial XGal screen. Nucleotide sequencing, using the chain termination method (Ausubel *et al.*, 1989), of the yeast DNA inserts in the plasmids showed that all but one insert encoded Gal4. This insert was found to encode a ~170-residue C-terminal fragment of the 1,950-residue Ubr1 protein (plasmid pKM1312, the exact number of Ubr1 residues encoded by the pKM1312 insert is not given because the corresponding sequencing gel was unreadable close to the sequencing primer site; data not shown). In a negative control, pKM1312 was transformed into the GGY1::171 strain carrying pEE5, a plasmid that expressed a fusion of the DNA-binding domain of Gal4 to Snf1, a protein presumably unrelated to Ubc2 (Yang *et al.*, 1992). As expected, these transformants yielded white colonies on XGal plates.

RESULTS

The Acidic C-terminal Region of Ubc2 Is Required for Degradation of N-end Rule Substrates—Degradation of proteins by the N-end rule pathway was assayed *in vivo* using derivatives of *E. coli* β-galactosidase (βgal) as model substrates. In eukaryotes, Ub fusions such as Ub-*X*-βgal are precisely deubiquitinated either *in vivo* or in cell-free extracts by Ub-specific processing proteases to yield *X*-βgal test proteins bearing the desired residue *X* at the N-terminus (Bachmair *et al.*, 1986; Gonda *et al.*, 1989). In contrast to the function of Ub in protein degradation, the role of Ub in these engineered Ub fusions is to allow the *in vivo* generation of otherwise identical proteins bearing different N-terminal residues. Depending on the identity of *X*, the *X*-βgal proteins are either long-lived or metabolically unstable, with destabilizing N-terminal residues conferring short half-lives on the corresponding *X*-βgals (Bachmair *et al.*, 1986; Tobias *et al.*, 1991). This N-terminal degradation signal (the N-degron) is manifested as the N-end rule.

Previous work (Dohmen *et al.*, 1991a) has shown that the normally short-lived N-end rule substrates such as Arg-βgal are long-lived in *S. cerevisiae* strains lacking the *UBC2* gene. One feature of the 172-residue Ubc2 Ub-conjugating enzyme is its highly acidic 23-residue C-terminal region which contains 20 acidic residues (Asp or Glu) (Fig. 1*A*, Reynolds *et al.*, 1985). The acidic "tail" of Ubc2 is required for some but apparently not for all of the Ubc2 functions. In particular, strains whose mutant Ubc2 protein lacks the acidic tail are unable to sporulate (as homozygous *ubc2* diploids), but they are nearly wild-type in their ability to carry out DNA repair and induced mutagenesis, in marked contrast to congenic *ubc2Δ* strains (Morrison *et al.*, 1988).

Given this earlier evidence, we asked whether tailless variants of Ubc2 are active in the N-end rule pathway. Otherwise identical plasmids expressing either the wild-type Ubc2 or its C terminally truncated derivatives (Fig. 1*A*) were transformed into a *ubc2Δ* yeast strain that expressed either Arg-βgal (Ub-

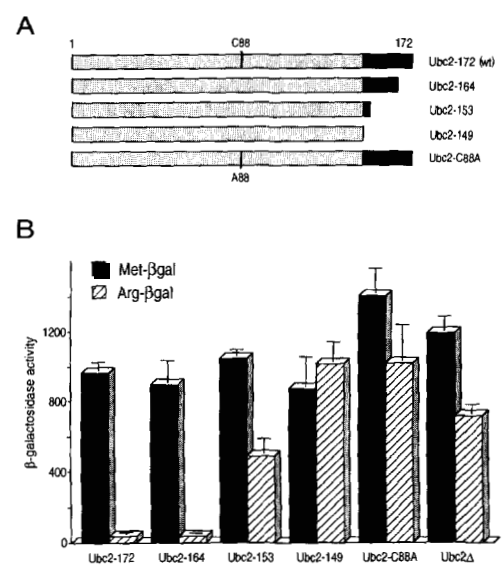

FIG. 1. **UBC2 alleles and their activity in the N-end rule pathway.** *A*, schematic representation of the 172-residue Ubc2, its C-terminally truncated derivatives (*Ubc2–164*, *Ubc2–153*, and *Ubc2–149*), and the enzymatically inactive Ubc2-C88A, in which the active-site Cys-88 has been replaced by Ala (*filled portions* of the *rectangles* denote the 23-residue polyacidic tail). See also main text. *B*, levels of βgal activity in the *S. cerevisiae* strain BBY67 (*ubc2Δ*) carrying plasmids pUb23M or pUb23R that expressed Ub-Met-βgal (*black bars*) or Ub-Arg-βgal (*striped bars*) from the galactose-inducible *GAL10* promoter (Bachmair *et al.*, 1986), and also either a low-copy (*CEN*-based) plasmid YCplac33 (vector alone; denoted as *ubc2Δ*) or similar plasmids pR67 (Morrison *et al.*, 1988), pR617, pR616, pR615, and pR647 expressing, respectively, Ubc2–172 (wt), Ubc2–164, Ubc2–153, Ubc2–149, and Ubc2-C88A (see also "Experimental Procedures" and Table I). Values shown are the means of three duplicate measurements with three independent transformants. Standard deviations are indicated above the *bars*.

Arg-βgal) or Met-βgal (Ub-Met-βgal) (see the legend to Fig. 1). Arg is a type 1 primary destabilizing residue in the N-end rule, whereas Met is a stabilizing residue (see Introduction). Metabolic stabilities of Arg-βgal and Met-βgal were compared by determining their intracellular concentrations (Fig. 1*B*). Previous work (Bartel *et al.*, 1990; Balzi *et al.*, 1990; Baker and Varshavsky, 1991; Dohmen *et al.*, 1991a) has shown that the steady state level of an *X*-βgal protein in yeast cells is a sensitive indicator of its metabolic stability; compare, for example, the levels of Met-βgal ($t_{1/2}$ > 20 h) and Arg-βgal ($t_{1/2}$ of ~3 min) (Bachmair *et al.*, 1986) expressed from identical vectors in cells that also expressed the wild-type *UBC2* gene (Fig. 1*B*, *Ubc2–172*). However, in the congenic *ubc2*Δ cells, the level of Arg-βgal was much higher, close to that of Met-βgal (Fig. 1*B*, *Ubc2*Δ), in agreement with earlier findings (Dohmen *et al.*, 1991a).

As can be seen from the data in Fig. 1*B*, the shortening of the 23-residue acidic tail of Ubc2 by 8 residues (yielding Ubc2–164) did not impair the ability of Ubc2 to support the degradation of Arg-βgal, whereas the Ubc2–153 derivative which lacked all but 4 residues of the acidic tail was severely impaired in this function (Fig. 1*B*, *Ubc2–164 versus Ubc2–153*). The residual activity of Ubc2–153 in the N-end rule pathway could be eliminated by deleting the 4 remaining residues of the acidic tail (Fig. 1*B*, *Ubc2–149 versus Ubc2–153*).

The level of the normally short-lived Arg-βgal in cells expressing the entirely tailless Ubc2–149 was indistinguishable from the level of the normally long-lived Met-βgal (Fig. 1*B*, *Ubc2–149*). In contrast, the level of Arg-βgal was significantly (and reproducibly) lower than that of Met-βgal in *ubc2*Δ cells (Fig. 1*B*, *Ubc2*Δ). Cells lacking Ubc2 are known to contain elevated levels of other Ub-conjugating (*E2*) enzymes, in particular Ubc4 (data not shown). This apparently "reciprocal" cross-regulation among *E2* enzymes suggests the following explanation for a paradoxically higher residual activity of the N-end rule pathway in the total absence of Ubc2 than in the presence of the tailless Ubc2. First, it is assumed that at least some of the *E2* enzymes other than Ubc2 have a weak but significant affinity for the *UBR1*-encoded N-recognin. Second, it is assumed that expression of either the wild-type Ubc2 or its tailless derivative such as Ubc2–149 down-regulates the expression of the postulated "cross-complementing" *E2* enzymes. This model could account for the above results (Fig. 1*B*, *Ubc2–149 versus Ubc2*Δ), because a (hypothetical) cross-complementing *E2* enzyme would be induced in a *ubc2*Δ strain but not in a *ubc2–149* strain. Examples of at least partial functional complementation between different *E2* enzymes in yeast have been reported previously. For instance, overexpression of the yeast *UBC5* gene partially complements proteolytic defects of the *ubc4*Δ mutant (Seufert and Jentsch, 1990).

The Acidic C-terminal Region of Ubc2 Is Required for High Affinity Binding of Ubc2 to N-recognin—Earlier coimmunoprecipitation experiments have shown that the *UBR1*-encoded N-recognin is physically associated with Ubc2 (Dohmen *et al.*, 1991a). We asked whether a Ubr1·Ubc2 complex that is stable enough to be detectable in a coimmunoprecipitation assay requires the presence of the acidic C-terminal region in Ubc2. A modified Ubr1 protein used in these experiments had its C-terminus extended with a 9-residue sequence derived from hemagglutinin (ha) of influenza virus and containing an epitope (the ha tag) recognizable by the monoclonal antibody 12CA5 (Bartel *et al.*, 1990; Dohmen *et al.*, 1991a). The epitope-tagged Ubr1 (Ubr1-ha) was functionally active, in that the *UBR1-ha* gene and the unmodified *UBR1* gene were indistinguishable in their ability to complement the *ubr1*Δ mutant (Bartel *et al.*, 1990). When overexpressed from the *ADH1* promoter on a high copy plasmid, the ~226-kDa Ubr1-ha protein could be immunoprecipitated from [^{35}S]methionine-labeled cell extracts with the anti-ha monoclonal antibody (Fig. 2*A*, *lane b*; compare with *lane a*) (*dots* in Fig. 2*A* mark the two Ubr1-unrelated yeast proteins that cross-reacted with the anti-ha monoclonal antibody; these proteins were also present in cells lacking the *UBR1* and *UBR1-ha* genes, and in addition almost comigrated with Ubr1-ha upon SDS-PAGE; see also Bartel *et al.* (1990) and Dohmen *et al.* (1991a)).

Extracts were prepared from [^{35}S]methionine-labeled *ubr1*Δ *ubc2*Δ cells transformed with either a control plasmid (vector alone), a plasmid expressing Ubr1-ha, or a plasmid expressing the wild-type Ubc2 or its mutated derivatives. The control

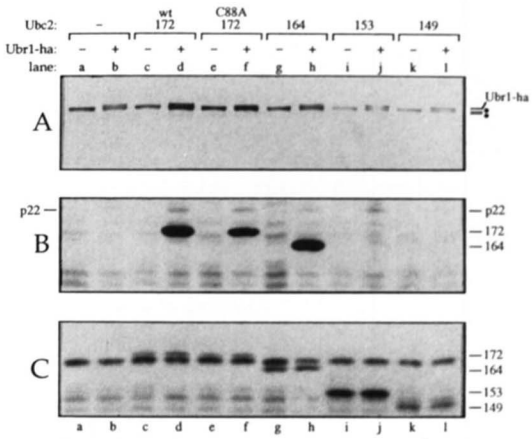

FIG. 2. **The polyacidic tail of Ubc2 is required for high affinity binding of Ubc2 to N-recognin.** Extracts were prepared from exponentially growing, [^{35}S]methionine-labeled BBY68 (*ubr1*Δ *ubc2*Δ) cells that had been transformed with one of the following high copy (2μ-based) plasmids: YEplac195ADH (vector alone); pSOB44 (expressing the ha epitope-tagged Ubr1-ha from the *ADH1* promoter (Bartel *et al.*, 1990)); pADHUBC2, pJD647, pJD617, pJD616, and pJD615 (the YEplac195ADH-based plasmids expressing, respectively, Ubc2–172 (wt), Ubc2-C88A, Ubc2–164, Ubc2–153 and Ubc2–149 from the *ADH1* promoter) (see "Experimental Procedures"). Extracts from ^{35}S-labeled cells (~1 × 10^7 acid-insoluble counts/minute/extract) carrying either the control plasmid (vector alone, labeled − atop the *lanes a, c, e, g, i,* and *k*) or the plasmid expressing Ubr1-ha (labeled + atop the *lanes b, d, f, h, j,* and *l*) were mixed either with a labeled extract from ^{35}S-labeled control cells (carrying vector alone; *lanes a* and *b)* or with extracts from ^{35}S-labeled congenic cells carrying plasmids that expressed Ubc2 and its derivatives (~0.75 × 10^7 acid-insoluble counts/minute/extract): Ubc2–172 (wild type) (*lanes c* and *d*); Ubc2-C88A (*lanes e* and *f*); Ubc2–164 (*lanes g* and *h*); Ubc2–153 (*lanes i* and *j*); and Ubc2–149 (*lanes k* and *l*). After removing a portion of each sample for the analysis of total protein, the mixed extracts were subjected to immunoprecipitation with a monoclonal antibody to the ha epitope, followed by SDS-PAGE (13%) and fluorography (see "Experimental Procedures"). *A*, A 3-h fluorographic exposure of an upper portion of the gel that shows the immunoprecipitated ~226-kDa Ubr1-ha (*lanes b, d, f, h, j,* and *l*). *Dots* mark two unrelated yeast proteins (present in all lanes) that cross-react with the anti-ha antibody (Dohmen *et al.*, 1991a). *B*, a 48-h fluorographic exposure of a lower portion of the same gel as in *A*. Positions of the coimmunoprecipitated 172-residue (20 kDa) Ubc2–172 (wild-type), Ubc2-C88A, and the 164-residue Ubc2–164 are indicated. Also denoted is the position of a ~22-kDa protein (*p22*) that coimmunoprecipitated with Ubr1-ha irrespective of the presence or absence of Ubc2 in the mixed extracts (this panel and data not shown; see also main text). *C*, the same molecular mass range as in *B*, in an identically run gel that was loaded with samples (1% of total ^{35}S counts/minute) from each of the mixed extracts *before* immunoprecipitation, confirming the approximate equimolarity of Ubc2 variants in the initial extracts. Positions and lengths of Ubc2–172 and its C terminally truncated derivatives are indicated by *numbers* on the *right*.

extract (lacking both Ubr1-ha and Ubc2 or its derivatives) was processed for immunoprecipitation with the anti-ha monoclonal antibody either alone (Fig. 2, *A* and *B*, *lane a*) or after having been mixed either with the extract containing Ubr1-ha (Fig. 2, *A* and *B*, *lane b*) or with the extract containing Ubc2 (or its derivatives) (Fig. 2, *A* and *B*, *lanes c, e, g, i,* and *k*). Each of the Ubc2-containing extracts was also mixed with the extract containing Ubr1-ha, and the mixed extracts were incubated with the anti-ha monoclonal antibody (Fig. 2, *A* and *B*, *lanes d, f, h, j,* and *l*). The antibody precipitated the Ubr1-ha protein from all samples that contained Ubr1-ha (Fig. 2*A*, *lanes b, d, f, h, j,* and *l*). Crucially, the antibody did not precipitate proteins the size of Ubc2 (19.7 kDa) or its derivatives from samples containing Ubc2 but lacking Ubr1-ha (Fig. 2, *A* and *B*, *lanes c, e, g, i,* and *k*). However, immunoprecipitation from the sample containing *both* Ubr1-ha and the wild-type Ubc2 protein yielded not only Ubr1-ha but also a protein the size of Ubc2 (Fig. 2, *A* and *B*, *lanes d*; compare with *lane c*), confirming the earlier finding that the wild-type Ubc2 is physically associated with Ubr1 (Dohmen *et al.*, 1991a).

Similar assays with Ubr1-ha and the C-terminally truncated Ubc2 derivative Ubc2–164, which lacks a portion of the acidic tail but is fully active in the N-end rule pathway (Fig. 1*B*, *Ubc2–164*), also resulted in the coimmunoprecipitation of Ubc2–164 and Ubr1-ha (Fig. 2, *A* and *B*, *lane h*; compare with *lane g*). The same experiment was carried out with more extensively truncated Ubc2 derivatives, Ubc2–153 and Ubc2–149. The former lacks all but 4 residues of the acidic tail and is nearly inactive in the N-end rule pathway, while Ubc2–149 lacks the entire acidic tail and has no detectable activity in the pathway (Fig. 1*B*, *Ubc2–153* and *Ubc2–149*). Neither Ubc2–153 nor Ubc2–149 were coimmunoprecipitated with Ubr1-ha, in contrast to either the wild-type Ubc2 or Ubc2–164 (Fig. 2, *A* and *B*, *lanes j* and *l*; compare with, for instance, *lane h*).

We conclude that a portion of the 23-residue acidic tail of Ubc2 that is longer than 4 residues but shorter than 16 residues is sufficient for both a high affinity (coimmunnoprecipitation assay-detectable) binding of Ubc2 to Ubr1 and for the function of Ubc2 in the N-end rule pathway. Regions of the Ubc2 enzyme other than the acidic tail may also be involved in functionally relevant interactions with N-recognin, or in other N-end rule-related functions, because neither the Ubc3 (Silver *et al.*, 1992; Kolman *et al.*, 1992) nor the Ubc4 Ub-conjugating enzyme that have been modified by the addition of the Ubc2-specific acidic tail could complement the N-end rule defect of a *ubc2Δ* strain.[3]

N-recognin Is Bound to a ~22-kDa Protein—The anti-ha antibody precipitated not only the ~226-kDa Ubr1-ha and the ~20-kDa Ubc2 (see above), but also a protein with an apparent molecular mass of ~22 kDa, termed p22 (Fig. 2*B*, *lanes b, d, f, h, j,* and *l*). The presence of p22 in an anti-ha immunoprecipitate required the presence of Ubr1-ha in the initial extract but did not depend on the presence of Ubc2 (data not shown and Fig. 2*B*, *lanes b* and *d*; compare with *lanes a* and *c*). Thus, the apparent association of p22 with N-recognin does not require an intact N-recognin · Ubc2 complex. The possibility that p22 is a C-terminal proteolytic fragment of Ubr1-ha that bears the ha tag and is therefore immunoprecipitable with the anti-ha antibody was tested by constructing a Ubr1 variant that bore two consecutive C-terminal ha tags.[4] Immunoprecipitation from an extract containing Ubr1-ha-ha should have resulted in a detectable (~1.5 kDa) increase in the size of the p22 protein if it were a C-terminal (ha-bearing) fragment of Ubr1-ha-ha. No such increase was observed (data not shown), suggesting that p22 is a distinct Ubr1-associated protein.

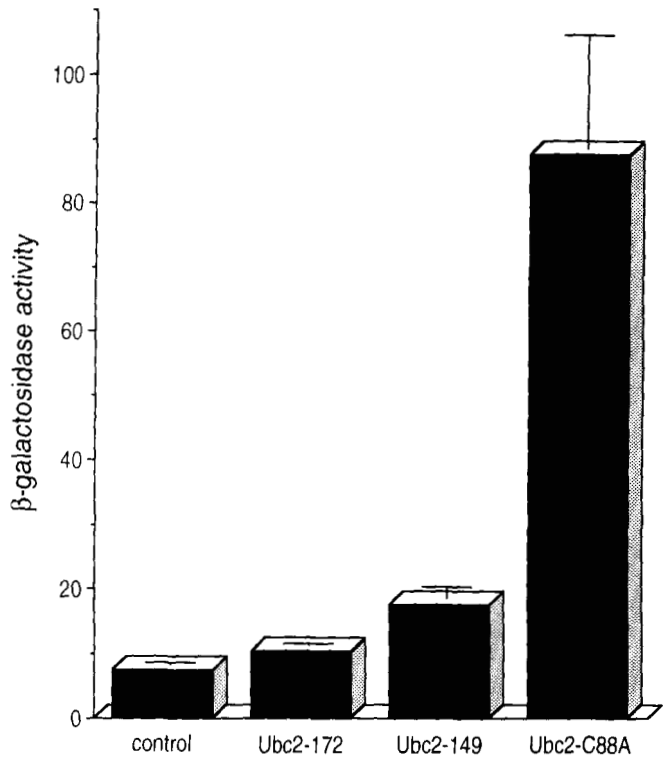

FIG. 3. **The inactive Ubc2-C88A derivative of the Ubc2 Ub-conjugating enzyme is an inhibitor of the N-end rule pathway.** Levels of βgal activity in the *S. cerevisiae* stain YPH500 (*UBR1 UBC2*) carrying the *LEU2*-based, high copy plasmid pRL2 that expressed Ub-Arg-βgal (Bartel *et al.*, 1990, and data not shown), and also either the plasmid YEplac195ADH (vector alone; *control*) or its derivatives (pADHUBC2, pJD615, and pJD647) that expressed, respectively, Ubc2–172 (wild-type), Ubc2–149, and Ubc2-C88A from the *ADH1* promoter (see also "Experimental Procedures" and Table I). Values shown are the means of four measurements carried out with four independent transformants. Standard deviations are shown above the *bars*.

A Dominant Negative Allele of UBC2—Cys-88, the only Cys residue in the Ubc2 Ub-conjugating enzyme, has been shown to be the site of Ub thioester formation; replacement of Cys-88 in Ubc2 with either Ala or Val eliminates the Ub-conjugating activity of Ubc2 and renders it biologically inactive (Sung *et al.*, 1990). As could be expected from these earlier findings, the Ubc2-C88A (Cys→Ala) derivative was found to be inactive in the N-end rule pathway (Fig. 1*B*, *Ubc2-C88A*). However, in contrast to the effect of overexpressing the tailless Ubc2–149 (which does not bind to N-recognin and is also inactive in the N-end rule pathway; see above), overexpression of Ubc2-C88A in wild-type (*UBC2*) cells resulted in strong inhibition of the N-end rule pathway (Fig. 3). No such inhibition was observed upon overexpression of the wild-type *UBC2* (Fig. 3). A likely reason for the difference between the effects of overexpressing Ubc2–149 and Ubc2-C88A is indicated by the finding that Ubc2-C88A, unlike Ubc2–149, retains the ability of the wild-type Ubc2 to form a high affinity complex with the *UBR1*-encoded N-recognin (Fig. 2, *A* and *B*, *lane f*; compare with *lane l*).

[3] R. J. Dohmen, K. Madura, and A. Varshavsky, unpublished data (a plasmid expressing a derivative of Ubc3 that bore the Ubc2-specific acidic tail was kindly provided by M. Ellison, University of Alberta, Edmonton, Canada).

[4] R. J. Dohmen, N. Schnell, and A. Varshavsky, unpublished data.

Thus, *ubc2-C88A* is a dominant negative allele of the *UBC2* gene, whose interference with the N-end rule pathway is likely to result from competition between the wild-type Ubc2 and Ubc2-C88A for binding to N-recognin. Although the Ubc2 regions in contact with N-recognin are unlikely to be confined to the acidic tail of Ubc2 (see above), the overall affinity of "non-tail" Ubc2 regions for N-recognin is at most low: Ubc2-149 is not coimmunoprecipitated with N-recognin (Fig. 2), and the inhibition of the N-end rule pathway by Ubc2-149 is much weaker than the inhibition by Ubc2-C88A (Fig. 3, compare *Ubc2-149* with *Ubc2-C88A*).

Ubc2-C88A, which lacks the thioester-forming Cys residue of the wild-type Ubc2, cannot accept Ub from the Ub-activating (*E*1) enzyme (Sung *et al.*, 1990). The apparently undiminished affinity of Ubc2-C88A for N-recognin (see above) suggests that interactions between N-recognin and Ubc2 do not involve the thioester-bound Ub moiety of the wild-type Ubc2 (at the *in vivo* concentrations of Ub, Ubc2, and the *UBA1*-encoded *E*1 enzyme, the bulk of Ubc2 is likely to bear the thioester-bound Ub moiety (Pickart, 1988)).

Ubc2-interacting Domain of N-recognin—To identify the domains of N-recognin that interact with the Ubc2 Ub-conjugating enzyme *in vivo*, we have used the "two-hybrid" interaction cloning technique of Fields and Song (1989). In this method, the expression of the *E. coli lacZ* reporter gene from the *GAL4*-dependent P_{GAL1} promoter in *S. cerevisiae* is designed to require the reconstitution of Gal4 activity from two unlinked but interacting protein domains. Specifically, the DNA-binding domain of the Gal4 protein is expressed in yeast as a fusion whose C-terminal portion is a polypeptide of interest. These cells are transformed with a library in which quasi-randomly generated DNA fragments (from yeast or other species) are fused to a fragment that encodes the Gal4 transcription activation domain. Transformants expressing a polypeptide from the library that has a significant affinity to the polypeptide of interest can be identified as those that induce their resident P_{GAL1}-*lacZ* reporter gene because the DNA-binding and activation domains of Gal4 are brought into spatial proximity through the interaction of polypeptides fused to each domain (Fields and Song, 1989; Chien *et al.*, 1991; Chevray and Nathans, 1992; Yang *et al.*, 1992).

The *S. cerevisiae* strain GGY1::171, which lacks both *GAL4* and *GAL80* genes and contains an integrated P_{GAL1}-*lacZ* reporter construct (Chien *et al.*, 1991), was transformed with the plasmid pKM1210-1 which expressed the Gal4 DNA-binding domain fused to the N-terminus of the full-length Ubc2. The resulting strain, KMY528, was transformed with pGAD-based DNA libraries that expressed the DNA-binding domain of Gal4 fused (in all three frames) to partial *Sau*3A fragments of the yeast genomic DNA (Chien *et al.*, 1991; see "Experimental Procedures"). 14 *lacZ*-expressing transformants were identified among ~1.5 × 10^5 colonies screened. While most of the pGAD plasmids from the positive transformants carried the intact *GAL4* gene (the pGAD libraries were derived from a *GAL4* yeast strain), one transformant was found to express a ~170-residue C-terminal fragment of N-recognin (Ubr1) fused to the Gal4 activation domain. The corresponding pGAD-based plasmid, termed pKM1312, reproducibly activated the expression of the P_{GAL1}-*lacZ* reporter gene upon retransformation of the KMY528 strain (GGY::171 carrying pKM1210-1). In a control experiment (data not shown), the same plasmid did not activate the expression of P_{GAL1}-*lacZ* in GGY1::171 carrying the plasmid pEE5, which expressed the DNA-binding domain of Gal4 fused to a region of a presumably unrelated protein Snf1 (Yang *et al.*, 1992). We conclude that a C-terminal region of N-recognin that encompasses approximately 10% of this 1,950-residue protein contains a Ubc2-interacting domain.

Regulatory Interactions between UBC2 and UBR1—In the course of earlier experiments (Dohmen *et al.*, 1991a), it was noticed that Ubr1 and Ubc2 could not be overproduced simultaneously within the same yeast cells. It was subsequently found that the levels of *UBR1* mRNA (expressed from the strong P_{ADH1} promoter on a high copy plasmid) were high in the absence of *UBC2* expression (in *ubc2*Δ cells) but much lower if cells carried a high copy plasmid overexpressing *UBC2* from the P_{ADH1} promoter. In addition, the copy number of a *UBR1*-expressing plasmid was decreased in the presence of a plasmid expressing *UBC2* but not in the presence of an otherwise identical plasmid lacking *UBC2*, suggesting that simultaneous overexpression of *UBR1* and *UBC2* is cytotoxic.[5]

To verify and analyze this effect, we constructed a high copy plasmid that expressed *UBR1* from the copper-inducible P_{CUP1} promoter, as well as a low copy (*CEN*-based) plasmid that expressed *UBC2* from the galactose-inducible P_{GAL1} promoter. Both plasmids were transformed into the *ubr1*Δ *ubc2*Δ strain KMY618, yielding the strain KMY633 (Table I), which was propagated in the raffinose-containing SR medium in the absence of added copper ions, conditions which do not induce the P_{GAL1} and P_{CUP1} promoters. (Induction of the P_{GAL1} promoter by galactose in cells that have been growing in the presence of raffinose is much faster than in cells that have been growing in the presence of dextrose (Griggs and Johnston, 1991; Johnston, 1987).)

The strain KMY633 was grown in SR medium containing 0.1 mM $CuSO_4$ (conditions that induce P_{CUP1}-*UBR1* but not P_{GAL1}-*UBC2*) to a midexponential phase ($A_{600} \leq 1$), followed by the addition of galactose to a final concentration of 3% (conditions that induce P_{GAL1}-*UBC2* as well). RNA was isolated from cells either immediately before or (at hourly intervals) after the addition of galactose, followed by Northern analysis with *UBR1* and *UBC2* hybridization probes (Fig. 4). While no expression of *UBC2* was detectable in SR medium, *UBC2* mRNA accumulated to a high level within 1 h after the addition of galactose (Fig. 4*A*, *UBC2*; compare *lanes a* and *b*). Conversely, the level of *UBR1* mRNA was high in SR medium but decreased by approximately 2.5-fold within 1 h after the addition of galactose, and remained low afterward (Fig. 4*A*, *UBR1*; compare *lanes a* and *b-d;* see also Fig. 4*D*). The rapid decrease of *UBR1* mRNA level upon induction of *UBC2* is likely to be caused by metabolic destabilization of *UBR1* mRNA, inasmuch as both *UBC2* and *UBR1* were expressed from heterologous promoters that are active in a copper-supplemented, galactose-containing medium. (Similar results were obtained when *UBR1* was expressed from the P_{ADH1} promoter or from its natural (P_{UBR1}) promoter (data not shown).) Moreover, since the doubling time of the KMY633 cells in this medium is approximately 3 h at 30 °C (data not shown), the rapidity of the observed decrease in the level of *UBR1* mRNA (Fig. 4, *A* and *D*) is unlikely to result from dilution of this mRNA due to cellular growth. A *UBC2*-induced destabilization of *UBR1* mRNA remains to be verified directly.

The inhibitory effect of *UBC2* expression on the level of *UBR1* mRNA was found to require the region that encodes the acidic C-terminal tail of Ubc2 (Fig. 1*A*): no significant decrease in the level of *UBR1* mRNA was observed upon induction of *ubc2-148* which encoded a tailless Ubc2 (Fig. 4, *B* and *D*). This result also confirmed that the transfer of cells

[5] K. Madura, B. Bartel, R. J. Dohmen, and A. Varshavsky, unpublished data.

TABLE I
S. cerevisiae strains

Name	Relevant markers	Source/comment
YPH500	*MATα ura3 leu2*	Sikorski and Hieter (1989)
JD47-13C	*MAT***a** *ura3 trp1 leu2*	This work
BBY67	*MATα ura3 ubc2Δ::LEU2*	Derivative of YPH500, Dohmen *et al.* (1991)
BBY68	*MATα ubc2Δ::LEU2 urb1Δ*	Dohmen *et al.* (1991)
KMY618	*MAT***a** *ura3 trp1 ubc2Δ::LEU2*	Derivative of JD47-13C, this work
KMY306	*MATα ubc2Δ::LEU2* + pUB23M	Derivative of BBY67, this work
KMY307	*MATα ubc2Δ::LEU2* + pUB23R	Derivative of BBY67, this work
GGY1::171	*gal4Δ gal80Δ ura3 his3 leu2 pGAL1*::lacZ	Fields and Song (1989)
KMY485	*ubc2Δ::LEU2* + pUB23M + pR67	Derivative of KMY306, this work
KMY487	*ubc2Δ::LEU2* + pUB23M + pR615	Derivative of KMY306, this work
KMY489	*ubc2Δ:LEU2* + pUB23M + pR616	Derivative of KMY306, this work
KMY491	*ubc2Δ::LEU2* + pUB23M + pR617	Derivative of KMY306, this work
KMY493	*ubc2Δ::LEU2* + pUB23M + pR647	Derivative of KMY306, this work
KMY486	*ubc2Δ::LEU2* + pUB23R + pR67	Derivative of KMY307, this work
KMY488	*ubc2Δ::LEU2* + pUB23R + pR615	Derivative of KMY307, this work
KMY490	*ubc2Δ::LEU2* + pUB23R + pR616	Derivative of KMY307, this work
KMY492	*ubc2Δ::LEU2* + pUB23R + pR617	Derivative of KMY307, this work
KMY494	*ubc2Δ::LEU2* + pUB23R + pR647	Derivative of KMY307, this work
KMY528	*gal4Δ gal80Δ* + pKM1210-1	Derivative of GGY1::171
KMY633	*ubc2Δ::LEU2* + pKM1167 + pKM1315	Derivative of KMY618, this work
KMY634	*ubc2Δ::LEU2* + pKM1166 + pKM1315	Derivative of KMY618, this work
KMY635	*ubc2Δ:LEU2* + pKM1167 + pKM1320	Derivative of KMY618, this work

to galactose-containing medium did not, by itself, cause the observed decrease in *UBR1* mRNA. To determine whether the *UBR1*-suppressing effect of Ubc2 also required a functional Ubr1 protein, we asked whether the induction of *UBC2* decreased the level of an mRNA produced from an insertion/frameshift *ubr1* allele that encoded a 170-residue N-terminal region of the 1,950-residue Ubr1 protein. No significant effect of *UBC2* on this *ubr1* allele was observed (Fig. 4, *C* and *D*), strongly suggesting that a functionally active Ubr1 protein is required for down-regulation of *UBR1* mRNA by *UBC2*.

A likely explanation of these findings (Fig. 4) is that the N-recognin · Ubc2 complex participates in the regulation of the *UBR1*-encoded N-recognin. For reasons considered above this regulation appears to involve an enhanced degradation of *UBR1* mRNA upon an increase in *UBC2* expression.

DISCUSSION

Our previous work has shown that the yeast N-recognin is physically associated with Ubc2, one of at least 10 Ub-conjugating enzymes in *S. cerevisiae* and an essential component of the yeast N-end rule pathway (Dohmen *et al.*, 1991a). Further analysis resulted in the following main findings.

(i) Both physical stability and functional activity of the N-recognin · Ubc2 complex require the presence of a 23-residue C-terminal region in Ubc2 that contains 20 acidic residues (Asp or Glu).

(ii) A Ubc2-interacting domain of the *UBR1*-encoded N-recognin is located within the 170-residue C-terminal region of the 1,950-residue N-recognin. Whether this is the only Ubc2-interacting region of N-recognin and whether this region interacts with the C-terminal acidic region of Ubc2 remains to be determined.

(iii) The *ubc2-C88A* gene, in which the codon encoding the active-site Cys of Ubc2 has been replaced by an Ala codon, acts as a dominant negative allele of *UBC2*, most likely as a result of competition between the wild-type Ubc2 and its enzymatically inactive variant for binding to N-recognin.

(iv) Expression of *UBC2* decreases the level of *UBR1* mRNA which encodes N-recognin. This effect requires the intact N-recognin-Ubc2 complex and is likely to result from enhanced degradation of *UBR1* mRNA upon an increase in *UBC2* expression.

Targeting Complex of the N-end Rule Pathway—The known components of the *S. cerevisiae* N-end rule pathway that mediate steps prior to or distinguishable from the actual proteolysis of a substrate are N-terminal amidase (Nt-amidase), encoded by the *NTA1* gene,[6] Arg-tRNA-protein transferase, encoded by the *ATE1* gene (Balzi *et al.*, 1990), N-recognin, encoded by the *UBR1* gene (Bartel *et al.*, 1990), the Ub-conjugating (*E*2) enzyme, encoded by the *UBC2* gene (Reynolds *et al.*, 1985; Jentsch *et al.*, 1987; Dohmen *et al.*, 1991a), and the Ub-activating (*E*1) enzyme, encoded by the *UBA1* gene (McGrath *et al.*, 1991). In addition to direct (immunoprecipitation-based) evidence for the physical association between N-recognin and Ubc2 (see "Results"), there is also circumstantial (overexpression-based) evidence for the existence of a ternary complex between N-recognin, Arg-tRNA-protein transferase, and Nt-amidase[6] (these latter components of the pathway modify N-termini of certain N-end rule substrates prior to their binding by N-recognin; see Introduction).

Proteins that participate in a complex metabolic transformation are often physically associated. The examples of a multienzyme assembly range from pyruvate dehydrogenase (synthesis of acetyl-CoA) to replisome (DNA replication), spliceosome (RNA splicing), ribosome (protein synthesis), and proteasome complexes (protein degradation). Mechanistic advantages of such assemblies stem from their increased fidelity due to often present editing capabilities, and also from the properties of processivity and channeling that are central to the functioning of multienzyme machines. For example, the association between N-recognin and the Ubc2 Ub-conjugating enzyme not only endows the latter with the ability to recognize a substrate that bears a destabilizing N-terminal residue but may also contribute to processivity of the Ubc2-mediated synthesis of a substrate-linked multi-Ub chain.

The targeting complex of the *S. cerevisiae* N-end rule pathway is likely to contain at least five components, Nta1, Ate1, Ubr1, and Ubc2 (whose subunit molecular masses are, respectively, 52, 58, 225, and 20 kDa), and also the at least transiently associated 114-kDa Uba1 (*E*1) enzyme, which must be bound to Ubc2 during the *E*1→*E*2 transfer of an activated Ub moiety. That this list is incomplete is suggested by the

[6] R. Baker and A. Varshavsky, manuscript in preparation.

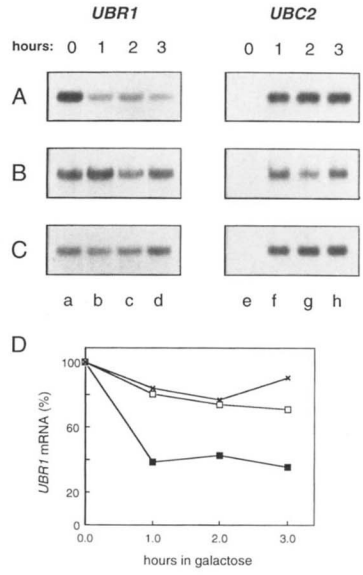

FIG. 4. **UBC2-mediated inhibition of UBR1.** Northern analysis of electrophoretically fractionated total RNA from the *S. cerevisiae* strain KMY618 (*ubr1Δ ubc2Δ*, Table I) transformed with a plasmid that expressed wild-type *UBC2* (or its derivative *ubc2–148*) from the galactose-inducible P_{GAL1} promoter, and also with a plasmid that expressed wild-type *UBR1* (or an insertion/frameshift allele of *UBR1*) from the copper-inducible P_{CUP1} promoter. *Numbers* at the *top* indicate time after the addition of galactose to cells growing in SR medium. The blots were hybridized simultaneously to *UBR1*- and *UBC2*-specific probes labeled with ^{32}P (see "Experimental Procedures"). Because of different signal intensities from the ~0.8-kb *UBC2* mRNA and the ~7-kb *UBR1* mRNA, the images shown were produced by autoradiographic exposures of different durations. *A*, RNA was isolated from exponentially growing cells ($A_{600} \leq 1$) carrying plasmids that expressed wild-type *UBR1* from the P_{CUP1} promoter and wild-type *UBC2* from the P_{GAL1} promoter (strain KM633, Table I) immediately before and 1, 2, or 3 h after the addition of galactose to cells growing in SR medium. Note the induction of the *UBC2* mRNA in the presence of galactose, and the concomitant decrease in the level of the *UBR1* mRNA. *B*, same as in *A* but with RNA from cells that expressed *ubc2–148* instead of the wild-type *UBC2* (strain KMY634, Table I). An underloaded RNA sample was the reason for apparently lower levels of both the *UBR1* and *UBC2* mRNAs at the 2-h point (verified by ethidium staining of the gel and by hybridization with a control *ACT1* probe; see *D*). *C*, same as in *A* but with RNA from cells that expressed an insertion/frameshift *ubr1* allele (encoding a short N-terminal fragment of Ubr1) instead of the wild-type *UBR1* (strain KMY635, Table I). *D*, the RNA blots in *A-C* were rehybridized with *ACT1*, the yeast actin gene (see "Experimental Procedures"). Hybridization signals from the *UBR1*, *UBC2*, and *ACT1* probes were recorded and quantified using PhosphorImager (Molecular Dynamics). The signals from the *UBR1* probe were normalized to those derived from the *ACT1* probe in each lane. Plotted are the normalized *UBR1* signals, with the signals at zero time point denoted as "100%". *Filled squares*, *UBR1/UBC2*; *open squares*, *UBR1/ubc2–149*; *crosses*, *ubr1* frameshift allele/*UBC2*.

finding of a ~22-kDa protein (distinct from the 20-kDa Ubc2) in association with N-recognin (see "Results") and by the earlier data indicating that a partially purified Arg-tRNA-protein transferase from rabbit reticulocytes is a ~360-kDa complex of several subunits of Arg-tRNA-protein transferase associated with several subunits of Arg-tRNA synthetase (Ciechanover *et al.*, 1988). If the yeast Arg-tRNA-protein transferase (encoded by *ATE1*) is organized similarly to its mammalian counterpart, the mass of the entire targeting complex in *S. cerevisiae* could be about 1,500 kDa. *In vivo*, this complex should be bound to the rest of proteasome at least some of the time. The complete proteolytic machine that implements the N-end rule is thus a strikingly diverse assembly of enzymes and binding factors whose combined size is comparable to those of ribosomal subunits.

Evolution of the Targeting Complex—Homologs of the *S. cerevisiae* Ubc2 enzyme are present in all eukaryotes examined, from yeasts to mammals. However, while a slightly shorter counterpart of the acidic C-terminal tail of the *S. cerevisiae* Ubc2 is also present in Ubc2 from the related budding yeast *Kluyveromyces lactis*,[7] this region is absent from the otherwise close homologs of Ubc2 such as the Rhp6 Ub-conjugating enzyme of the fission yeast *Schizosaccharomyces pombe* (Reynolds *et al.*, 1990), and from the *Drosophila*, rabbit, and human homologs of Ubc2 as well (Koken *et al.*, 1991a; Wing *et al.*, 1992; Schneider *et al.*, 1990; Koken *et al.*, 1991b).

In *S. cerevisiae*, the tailless derivative of Ubc2 is inactive in sporulation and in the N-end rule pathway but is able to support other Ubc2 functions such as DNA repair and induced mutagenesis at nearly wild-type levels (see "Results" and Morrison *et al.*, 1988). The sporulation defect in cells containing exclusively the tailless Ubc2 is unrelated to their lack of the N-recognin-Ubc2 complex because *S. cerevisiae* is capable of sporulation in the absence of N-recognin and hence in the absence of the N-end rule pathway (Bartel *et al.*, 1990). Thus, it is likely that the acidic tail of Ubc2 is also required for an interaction with an unknown Ubc2-specific recognin whose function is essential for sporulation in *S. cerevisiae*.

The naturally tailless Ubc2 homolog Rhp6 of *S. pombe* was shown to complement the known phenotypes of *ubc2Δ S. cerevisiae* except for its defect in sporulation (complementation of the N-end rule defect has not been tested) (Reynolds *et al.*, 1990). Conversely, the tailless derivative of the *S. cerevisiae* Ubc2 enzyme could complement all of the tested defects of an *rhp6Δ S. pombe* mutant, including its sporulation defect (Reynolds *et al.*, 1990). Either the wild-type or tailless Ubc2 of *S. cerevisiae* could function as an N-end rule-mediating enzyme in an extract from rabbit reticulocytes (Sung *et al.*, 1992).

The tailless Ubc2 might have preceded its acidic tail-containing variant in a lineage of organisms that yielded *S. cerevisiae* and *K. lactis*. In this model, N-recognin and Ubc2 of the *S. cerevisiae* lineage (but not the homologous protein pairs in predecessors of fission yeast and larger eukaryotes) coevolved in a way that resulted in a positively charged surface of a domain in N-recognin accommodating the multiple Asp and Glu residues of the acidic tail in Ubc2. Throughout this coevolution, Ubc2 would be expected to retain its affinities for other recognins (which mediate other functions of Ubc2) in ways that did not depend on the presence of an acidic tail in Ubc2. However, the converse interpretation, in which a tail-bearing Ubc2 and a correspondingly adapted N-recognin is the ancestral configuration, is also plausible, in part because nothing is known about the nature of selective pressures that could underlie a tail-related coevolution of Ubc2 and N-recognin in some but not in all species.

Regulatory Interactions in the N-end Rule Pathway—The intact N-recognin · Ubc2 complex was shown to be required for an observed decrease in the level of *UBR1* mRNA (which encodes N-recognin) upon an increase in *UBC2* expression. A variety of evidence suggests that a decrease in the level of *UBR1* mRNA is brought about by a *UBC2*-mediated decrease in its metabolic stability (see "Results"). It remains to be seen whether this new effect of Ubc2 is mediated by the presently known function of the N-recognin · Ubc2 complex, *i.e.* by an N-end rule-mediated degradation of a short-lived protein that directly or indirectly stabilizes *UBR1* mRNA, or whether a proteolysis-independent mechanism is involved.

Expression of genes that encode interacting proteins is

[7] P. R. H. Waller and A. Varshavsky, unpublished data.

often coregulated to maintain an appropriate concentration of a multiprotein complex as well as correct stoichiometries of its components. The effect of *UBC2* expression on the level of *UBR1* mRNA (see "Results") is a likely example of such a regulation. The expression of *S. cerevisiae* genes encoding Nt-amidase and Arg-tRNA-protein transferase appears to be coregulated as well, presumably to maintain correct stoichiometry of these interacting enzymes.[6] The evidence for coregulation includes the presence of common nucleotide sequence motifs in upstream regions of genes that encode components of the N-end rule pathway.[6]

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